A novel avian homologue of CD72, chB1r, downmodulates BCR-mediated activation signals

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Abstract

The avian B cell differentiation antigen chB1 is a C-type lectin membrane protein most homologous to mammalian CD72. Here, we report a new chB1-related gene, chB1r, that is located 18 kb away the chB1 gene. The cytoplasmic domain of chB1r protein contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs: ITIM1 and 2), which are identical to those found in CD72, whereas chB1 lacks the second ITIM2. Although chB1 expression is restricted to the bursa and an immature B cell line, chB1r is highly expressed in the bursa, spleen and both immature and mature B cell lines, a pattern that parallels CD72 expression. SHP-1 and Grb2 interact with phosphorylated tyrosine residues within chB1r ITIM1 and ITIM2, respectively. By contrast, ITIM1 of chB1 does not interact with SHP-1. Functional characterization using chB1r/chB1 double-deficient DT40 B cells demonstrated that ITIM1 in chB1r transduces a negative signal for BCR-mediated nuclear factor of activated T cells (NF-AT) activation and that ITIM2 attenuates this negative signal. This study has established chB1r as the genuine avian homologue of mammalian CD72, and revealed an opposing role for the two ITIMs through binding with SHP-1 and Grb2 for regulation of BCR-mediated NF-AT activation.

Introduction

CD72, a type II transmembrane protein of the C-type lectin family, is expressed predominantly in B lineage cells except for plasma cells (1, 2), where it serves to inhibit B cell antigen receptor (BCR)-mediated signaling (3). The cytoplasmic NH2-terminal region of CD72 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM; ITIM1 and 2) and an ITIM-like sequence (ITIM2). Upon tyrosine phosphorylation, SHP-1 and Grb2 are recruited to ITIM1 and ITIM2, respectively (4–6), which may be responsible for the negative regulatory role of CD72 in BCR-mediated B cell activation (7–9).

We previously identified an avian C-type lectin gene named chB1 that encodes a protein with a homology to mammalian CD72 (10, 11). Although the genomic structure of chB1 is very similar to that of mouse CD72 (11, 12), the expression patterns and functions of chB1 and CD72 are not (4–6). chB1 is expressed primarily on B cells in the bursa of Fabricius and the DT40 B cell line (10), both of which are in the immature stage of B cell development and undergo diversification of their Ig genes by gene conversion. In marked contrast to mammalian CD72, chB1 expression is decreased on mature B cells in the periphery and on corresponding cell lines. Although crosslinking of chB1 on bursal B cells and the DT40 cell line inhibits proliferation, no effect on BCR signaling could be demonstrated (10). This may be due to the absence of a motif corresponding to the ITIM2 of CD72 in the cytoplasmic domain of chB1. Thus, although related to mammalian CD72, chB1 appears not to be the actual homologue, which may be encoded by another gene. Indeed, we noticed a few faint bands in addition to the one that intensely hybridized with a chB1 cDNA probe in the Southern blot analysis of chicken genomic DNA (10). Thus, we embarked upon a search for the CD72 homologue in the chicken genome and present data indicating that chB1r is the genuine homologue of mouse CD72.
Methods

Antibodies and cell lines
The anti-chB1 (10) and anti-chicken IgM mAbs, M1 and M4 (13), were prepared from corresponding hybridomas. The following antibodies were purchased: anti-T7 epitope antibody from Novagen (Madison, MI, USA), anti-phosphotyrosine PY20 antibody conjugated with HRP from BD PharMingen (San Jose, CA, USA), anti-SHP-1 antibody from Upstate Laboratories (Charlottesville, VA, USA) and anti-Grb2 antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Avian leukosis virus-transformed chicken B cell lines, DT40, 293B9 and 249L4, and Marek’s Virus-transformed T cell lines, RP1 and JP2, were maintained in RPMI 1640 with 10% fetal bovine serum at 40°C in a humidified atmosphere.

Isolation of the chB1r genomic clone and cDNA clone
A 30-kb genomic clone containing the chB1 gene was previously isolated from a chicken genomic library constructed in the pWE15 cosmid vector (BD Clontech, Palo Alto, CA, USA) (11). After detailed restriction enzyme mapping of this genomic clone (see Fig. 1A), the 2.0-kb EcoRI fragment that hybridized with a full-length chB1 cDNA probe was sub-cloned into the pBluescript vector (Stratagene, La Jolla, CA, USA) for sequencing. Subsequently, the 4.4-kb EcoRI fragment adjacent to the 2.0-kb fragment was also sub-cloned into the plasmid vector and sequenced. The 4.4- and 2-kb fragments together contained the complete chB1r gene. A chB1r cDNA was amplified by 3’ rapid amplification of cDNA ends (RACE) PCR using an adaptor-ligated oligo-(dT)-primed cDNA library synthesized from bursa mRNA (Marathon cDNA amplification kit; BD Clontech) with a 3’ adaptor primer provided by the kit and a gene-specific primer based on the sequence corresponding to the region upstream of the predicted translation initiation codon (5′-TGC GAC AGA GCC ACC AGC AGC ACC-3′). The amplified 1-kb band was sub-cloned into the pCR2 vector (Invitrogen, Carlsbad, CA, USA) and sequenced. The coding region of chB1r DNA was also sub-cloned into pBluescript vector. DNA sequencing was performed using CEQ Dye Terminator Cycle Sequencing Kit with CEQ 2000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA). Homology searches were performed using the BLAST database at the National Center for Biotechnology Information. The CLUSTAL-W program was used for sequence alignment.

Reverse transcription–PCR
Total RNA was extracted from chicken tissue samples and cell lines by the acid guanidium–phenol method (10). Total RNA was also prepared from thymic CD4+ and CD4+CD8+ double-positive T cells, which were sorted after staining with FITC-labeled anti-CD4 and PE-labeled anti-CD8 antibodies (Southern Biotechnology Associates) by a FACSvantage (Becton Dickinson) with the aid of CELLQuestTM software. These RNAs were converted into single-stranded cDNA using SuperScriptII reverse transcriptase with oligo-(dT) primer (Invitrogen). The resultant cDNAs were subjected to PCR for chB1r, chB1 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA using the following specific primers: 5′-ATG GCC CAG AGC GTG CTC TAC AGC CTG-3′ and 5′-TCA CCC TTC GGC CAG CAG AGG AAA GAT CC-3′ for chB1r; 5′-ATG GCC CAA GAA GTC CTC TAC ACT GAC CTG-3′ and 5′-TCA TAT GAA TGG GGA GCT GGG CCC TGC-3′ for chB1 and 5′-ATT TGG CCG TAT TGG CCG CC-3′ and 5′-CAT AAG ACC CTC CAC AAT GCC-3′ for GAPDH.

Expression vectors
Point mutations (tyrosine to phenylalanine) in the ITIMs of chB1r and chB1 cDNAs were generated using a Quickchange site-directed mutagenesis kit (Stratagene). The cDNAs encoding chimeric molecules with the extracellular domain of chB1 and the intact or modified cytoplasmic domains of chB1r were constructed by PCR (14). After sequence confirmation, they were sub-cloned into a pCAT7neo expression vector (15). The pAct-βGal vector was kindly provided by T. Yagi (Osaka University). Expression vectors encoding mouse Lyn, SHP-1 and human Grb2 were provided by H. Nishizumi (The University of Tokyo), B. G. Neel (Beth Israel Deaconess Medical Center) and M. Tanaka (Hamamatsu Medical College), respectively.

Immunoprecipitation and immunoblot analysis
DT40 cells were transfected with 10 μg of either expression vector in a serum-free RPMI medium at a density of 105 cells per 400 μl per cuvette with a gene pulser apparatus (Bio-Rad Laboratories, Hercules, CA, USA) set at 250 V and 975 μF. After electroporation, the cells were transferred to complete RPMI medium and incubated at 40°C for 48 h. The resultant cells were either left unstimulated or stimulated with anti-chicken IgM antibody, M4 (11) or with anti-chB1, anti-chicken IgM (M1) or anti-chB1 plus anti-IgM with F(ab')2 fragment of rabbit anti-mouse IgG at 37°C for the indicated period of time, and then lysed at 2×105 cells ml-1 in lysis buffer containing 1% NP40, 10 mM Tris (pH 7.8), 150 mM NaCl, 2 mM EDTA, protease and phosphatase inhibitors, as previously described (15). Lysates were cleared by centrifugation, then immunoprecipitated with the indicated antibodies and protein G-Sepharose. Immunoprecipitates were then washed extensively in the lysis buffer, resolved by SDS-PAGE, and then transferred to nitrocellulose membranes. Blots were blocked with 3% BSA in TBST buffer [10 mM Tris (pH 7.9), 150 mM NaCl, with 0.05% Tween 20], and then incubated with the indicated antibodies, followed by a secondary antibody conjugated with HRP. Immunoreactive proteins were detected by ECL (Amersham Biosciences, Piscataway, NJ, USA). COS-7 cells (5×106) were transfected with various combinations of expression vectors using a TransIT-LT1 transfection reagent (Mirus Co., Madison, WI, USA). After incubation for 48 h, cell lysates were prepared as described above. In some experiments, blots were scanned and analyzed with the National Institutes of Health (NIH) image software. Results were expressed as fold induction over baseline and were normalized for loading.

Generation of chB1r, chB1 and chB1r/chB double-deficient DT 40 cells
The targeting vector for the chB1r gene locus was constructed by inserting a his-cassette (provided by T. Kurosaki, RIKEN, Tsuru) into the chB1r gene at an exon III, which corresponds to the transmembrane region. In the resultant vector
The his-cassette was flanked by 0.9 and 3.1 kb of chB1r genomic sequence on the 5' and 3' sides, respectively. The chB1r gene targeting vector was constructed using a 6-kb EcoRI–KpnI fragment containing exons 1–4. A 1.1-kb BglII–BamHI portion containing exon I was replaced with the neo-cassette (provided by T. Kurosaki). In this construct (pchB1r-neo), the neo-cassette was flanked by 2.0- and 3.0-kb chB1 genomic sequence on the 5' and 3' sides, respectively. To generate chB1r- or chB1-deficient DT40 cells, pchB1r-his or pchB1-neo was transfected into DT40 cells by electroporation using the condition described above, and transfectants were selected in the presence of histidinol (1.5 mg ml⁻¹) or G418 (2 mg ml⁻¹). Drug-resistant clones were screened for targeted integrations by Southern blot analysis. For the generation of chB1r/chB1 double-deficient DT40 cells, the targeting vector pchB1-neo was transfected into the chB1r-deficient DT40 cells. Clones were selected in the presence of G418, and screened for the targeted integration as described above. Evidence for null mutants of these knock-out DT40 cells was obtained by northern blot analysis (10). Briefly, RNA samples...
(10 μg) were electrophoresed in 1.2 M formaldehyde/1.2% agarose gel and transferred to nylon membranes. The blots were hybridized with cDNA probes corresponding to the 3′ portion of chB1r or chB1. Filters were stripped and rehybridized with chicken GAPDH cDNA probes to verify equal loading of RNA samples.

Luciferase assay

The chB1/chB1r double-deficient DT40 cells were transfected by electroporation with 10 μg of a luciferase reporter plasmid driven by seven tandem copies of the NF-AT response element taken from the mouse IL-2 gene promoter (a gift from K. Arai, Tokyo Metropolitan Institute of Medical Science), together with 15 μg of expression vectors encoding either chB1 or chB1/chB1r chimeric molecules. After 48-h culture, triplicates of 5 × 10⁵ viable cells were then stimulated with anti-chB1, anti-IgM (M1) or anti-chB1 plus anti-IgM with F(ab′)² fragment of rabbit anti-mouse IgG for 6 h and subsequently assayed for luciferase activity, as described (15). Light emission was measured in a Lumat LB9501 luminometer (Berthold, Wildbad, Germany).

Results

Genomic organization and the primary structure of the chB1r gene

Within a 28-kb chicken genomic DNA fragment, we identified a 2-kb EcoRI fragment approximately 18-kb 5′ of the chB1 gene that weakly hybridized with the chB1 cDNA probe (Fig. 1A and data not shown). DNA sequencing of this 2 kb and adjacent fragments revealed the presence of a novel gene encoding a C-type lectin that is related to chB1. This gene spans ~3 kb in the genome and has the same genomic orientation as the chB1 gene. To determine whether this chB1-related gene is transcribed, we carried out 3′ RACE PCR using adaptor-ligated oligo(dT)-primed cDNAs synthesized from bursal B cell mRNA with primers corresponding to the sequences of the adaptor and the putative 5′-untranslated region. This yielded a single amplified PCR product ~1 kb in size. Sequence analysis indicated that this cDNA encodes a novel protein with an open reading frame of 325 amino acids showing high homology to chB1 (Fig. 1C), which we named chB1r (chB1 related). BLAST analysis on the draft chicken genome sequence of a red jungle fowl showed that the several sequences with sufficient sequence identity to the chB1r genes to be detected. Among them, one is found in chromosome Z (NW_060764). However, we could not accurately confirm the positions of chB1r and chB1 genes, because the assembly of chromosome Z was still incomplete.

Comparison of the gene structure of chB1r with other C-type lectin genes indicates that organization of the chB1r gene is similar to chB1 and CD72. The sequence and locations of the exon/intron boundaries of chB1r are similar to those of chB1 and CD72, except for (i) both chB1r and chB1 genes lack an exon corresponding to the third exon of the CD72 gene. (ii) chB1r lacks the separate terminal exon encoding the 3′-untranslated region (Fig. 1B). This is the case for most C-type lectin family genes, such as rat asialoglycoprotein receptor type I (16), human CD23 (17) and rat Kupffer cell receptor (18) (Fig. 1B).

In this regard, chB1r retains a more prototypic gene structure of C-type lectin family members than do chB1 and CD72.

Comparison of amino acid sequence of chB1r with chB1 and CD72

Alignment of the chB1r, chB1 and CD72 protein sequences identified two regions of greatest similarity. The first is located in a membrane proximal extracellular domain that forms an α-helical coiled coil and is important for CD72 oligomerization (19). In this domain, chB1r contains four short consensus repeats of leucine residues with 14-amino acid spacing, whereas five and six repeats are found in chB1 and mouse CD72, respectively (Fig. 1C). The similarity between chB1r and chB1 is also evident in the transmembrane and cytoplasmic domains. Overall, amino acid similarities of the cytoplasmic and transmembrane domains between chB1r and chB1 are 63 and 78%, respectively. The cytoplasmic domains of chB1r and CD72 show low homology, but share identical ITIMs (ITIM1: 7YADL and ITIM2: 38YENV) locating at similar positions. On the other hand, chB1 contains the ITIM1 (7YDTL), but not the ITIM2.

In contrast to the high sequence similarities in the cytoplasmic, transmembrane and stalk domains of chB1r and chB1, their C-type lectin domains are more divergent. Nevertheless, the chB1r protein contains seven cysteine residues in its C-type lectin domain, which are conserved among chB1 and mouse CD72. Four of these cysteines presumably form two pairs of intrachain disulfide bonds (Cys-226–Cys-305 and Cys-284–Cys-297 in chB1r). Also conserved is the WIGL (aromatic–aliphatic–glycine–aliphatic) motif that is believed to play a key role in linking the two distinct hydrophobic cores of the C-type lectin domain (20).

chB1r is transcribed more broadly in chicken B lineage cells than chB1

We next examined the expression pattern of the chB1r gene in various chicken tissues by reverse transcription (RT)–PCR. In contrast to the exclusive expression of the chB1 mRNA in the bursa, chB1r mRNA was strongly expressed in bursa and modestly in spleen, thymus and peripheral blood leukocytes (Fig. 2A). Although chB1r mRNA was expressed in the thymus, we were unable to detect chB1r mRNA expression in sorted CD4⁺, CD8⁺ single-positive and CD4⁺CD8⁻ double-positive thymocytes (Fig. 2B). chB1r mRNA was detected in all of the B cell lines examined, but not in T cell lines, whereas chB1 mRNA was only found in the immature B cell line, DT40 (Fig. 2C), indicating that chB1r is expressed more broadly in chicken B lineage cells than chB1.

Association of SHP-1 and Grb2 with chB1r

Cross-linking of the BCR on mouse B cells induces tyrosine phosphorylation of CD72 (4, 5). We thus examined the phosphorylation status of chB1r as well as chB1 in DT40 cells. Expression vectors encoding T7 epitope-tagged wild-type chB1r or chB1 were transfected into DT40 cells, and transiently expressed proteins were immunoprecipitated with anti-T7 antibody. Tyrosine phosphorylation of CD72 (4, 5). We thus examined the phosphorylation status of chB1r as well as chB1 in DT40 cells. Expression vectors encoding T7 epitope-tagged wild-type chB1r or chB1 were transfected into DT40 cells, and transiently expressed proteins were immunoprecipitated with anti-T7 antibody. Tyrosine phosphorylation of CD72 (4, 5).
ITIMs are targets for BCR-associated protein tyrosine kinases, we generated mutant forms of chB1r and chB1 that contained either single (Y7F or Y38F) or double (Y7/38F) tyrosine to phenylalanine substitutions, and examined their tyrosine phosphorylation. Since the presence of the endogenous chB1 and chB1r in DT40 cells might hamper binding of the exogenous T7-tagged chB1r and chB1, we used COS-7 cells for these experiments. Both wild-type chB1 and chB1r were found to be tyrosine phosphorylated by Lyn, whereas Y7F and Y7/38F mutations abolished tyrosine phosphorylation of chB1 and chB1r, respectively (Fig. 3B). Although the Y7F mutant of chB1r was phosphorylated to the same extent as wild-type chB1r, the Y38F chB1r mutant displayed a severe reduction in phosphorylation. These results suggest that Tyr7 in chB1r is a target for Lyn-mediated phosphorylation, while Tyr38 is a major, but not exclusive, target in chB1r.

We next examined whether these chB1r tyrosine residues serve as binding sites for SHP-1 and Grb2 by transfecting COS-7 cells with the chB1r plasmids in various combinations with expression vectors encoding mouse SHP-1, Grb2 and Lyn. In the presence of Lyn, SHP-1 associated with wild-type and Y38F mutant form of chB1r, but not with the Y7F mutant of chB1r or wild-type chB1 (Fig. 3C). In contrast, Grb2 was associated with the wild-type and Y7F mutant of chB1r but not with the Y38F mutant of chB1r or chB1 (Fig. 3D). Furthermore, the association of chB1r with Grb2 was dependent of the integrity of the SH2 domain but not the SH3 domain of Grb2 (Fig. 3E), suggesting that the SH2 domain of Grb2 is associated with the phosphorylated Tyr38 of chB1r. These findings indicate that Lyn phosphorylates Tyr7 and Tyr38 in chB1r, which results in the recruitment of SHP-1 and Grb2 to the ITIM1 and the ITIM2, respectively. Although chB1 contains Lyn-phosphorylated Tyr7 in a motif similar to the chB1r ITIM1, this tyrosine residue does not serve as a SHP-1-binding site.

Co-ligation of BCR with chB1r inhibits BCR-induced NF-AT activation

To evaluate the function of chB1r as well as chB1 in BCR-mediated activation, we generated chB1r, chB1 and chB1r/chB1 double-deficient DT40 cells using a gene-targeting approach (Fig. 4). To disrupt the chB1r locus in DT40 B cells, we transfected a targeting construct pchB1r-his into parental DT40 B cells. Southern blot analysis indicated that 4 out of 28 histidinol-resistant clones had simultaneous targeting events on both alleles (Fig. 4A and data not shown). One of the chB1r-deficient DT40 clones was subsequently transfected with a pchB1-neo vector to disrupt the chB1r locus and was selected in the presence of G418. Among 36 drug-resistant clones screened, we obtained two clones that had both chB1 alleles simultaneously targeted (Fig. 4B and C, data not shown). Absence of chB1r and chB1 mRNA was verified by northern blot analysis (Fig. 4D) and the protein by flow cytometry in case of chB1r (data not shown). ChB1 single-deficient DT40 cells were also generated similarly (Fig. 4D). ChB1r-, chB1- and chB1r/chB1-deficient DT40 cells showed no gross abnormality in growth rate and surface expression of IgM (data not shown).

We used the chB1/chB1r-deficient DT40 cells to examine the function of chB1r in BCR-mediated NF-AT activation, one of important events resulting from BCR signaling (21). Since antibodies against chB1r were not available, we generated an expression vector encoding the extracellular portion of chB1 fused with the transmembrane and cytoplasmic portion of chB1r (Fig. 5A). These fusion proteins as well as wild-type chB1 were transiently expressed in chB1/chB1r-deficient DT40 cells and NF-AT activation upon co-ligation with anti-BCR and anti-chB1 mAbs was examined by a luciferase reporter assay. Co-ligation of chB1 or chB1Y7F with the BCR did not inhibit BCR-induced NF-AT activation (Fig. 5B and C), which is consistent with our finding that the ITIM in chB1 does not interact with SHP-1 (Fig. 3C). Remarkably, when the cytoplasmic portion of chB1 was replaced with that of chB1r (chB1/chB1r), co-ligation of chB1/chB1r with BCR completely abolished BCR-induced NF-AT activation, indicating that the cytoplasmic portion of chB1r delivers an inhibitory signal for BCR-mediated NF-AT activation (Fig. 5D). In contrast, the chB1/chB1rY7F mutant profoundly enhanced BCR-mediated NF-AT activation to a level comparable to that stimulated by phorbol myristate acetate plus ionomycin, suggesting that the remaining ITIM2–Grb2 interaction enhances BCR-mediated NF-AT activity (Fig. 5E). The Grb2-non-binding chB1/chB1rY38F mutant suppressed BCR-induced NF-AT activation after co-ligation with BCR (Fig. 5F). Furthermore, the chB1/chB1rY7/38F, which lacks binding with both SHP-1 and Grb2, did not alter BCR-mediated NF-AT activation (Fig. 5G). Consistent with the effect on BCR-induced NF-AT activation, co-ligation of the BCR with chB1/chB1rY38F mutant as well as chB1/chB1r reduced phosphorylation of the cytoplasmic tyrosines in chB1r whereas chB1/chB1rY7F mutant that lacks a SHP-1-binding
A novel avian homologue of CD72

Discussion

In the present study, we demonstrate that chicken B cells express two highly related genes, chB1r and chB1, both of which are homologous to the single CD72 gene found in mammals. Despite our previous speculation that chB1 is a CD72 homologue, we conclude from this study that chB1r is the genuine homologue, based on its protein structure, function and expression pattern.

Mouse CD72 has been shown to down-modulate BCR-mediated activation. The regulatory mechanisms exerted by CD72 are quite interesting, since the molecule recruits seemingly contradictory molecules, SHP-1 and Grb2. SHP-1 is known to negatively regulate BCR signaling, whereas Grb2 functions generally as a positive regulator. SHP-1 is associated with the phosphorylated tyrosine residue of ITIM1 and Grb2 binds to ITIM2 of CD72 (4–6). The chB1r protein contains ITIM1 and ITIM2 that are identical to those of CD72, whereas chB1 contains only ITIM1 (Fig. 2). The tyrosine residues in these ITIMs of chB1r as well as chB1 were tyrosine phosphorylated upon BCR stimulation in B cells and by Lyn in COS-7 cells (Fig. 3).
Furthermore, as is the case with CD72, the phosphorylated ITIM1 and ITIM2 in chB1r serve as binding sites for SHP-1 and Grb2, respectively. By contrast, the phosphorylated ITIM1 in chB1 failed to bind to SHP-1. The ITIM1 in chB1 differs one amino acid from chB1r and CD72: 7YTDL in chB1 and 7YADL in chB1r and CD72 (Fig. 2). This sequence difference may explain the differential accessibility of SHP-1 to chB1r and chB1.

We eliminated both chB1r and chB1 proteins from DT40 cells through gene targeting in order to independently examine the signal regulatory mechanisms of chB1r and chB1. Consistent with its inability to interact with SHP-1, chB1 failed to suppress BCR-induced NF-AT activation. In contrast, the chB1/chB1r chimeric molecule containing two ITIMs of chB1r completely abolished BCR-induced NF-AT activation. Furthermore, the mutant chimera lacking a Grb2-binding ITIM2 but retaining SHP-1-binding ITIM1 suppressed BCR-induced NF-AT activation, whereas the reverse mutant lacking the ITIM1 but retaining ITIM2 enhanced NF-AT activation, suggesting that chB1r negatively regulates BCR signaling via a dominant action of the ITIM1-bound SHP-1, but can also positively regulate signaling via ITIM2-bound Grb2 when SHP-1 is not recruited. This hypothesis was substantiated by the results of the mutant chimera lacking both ITIM1 and ITIM2, as the mutant has neither positive nor negative effects on BCR-induced activation of NF-AT. These functional aspects on chB1r are similar to those of CD72, in that ITIM1/SHP-1 and...
Fig. 5. The cytoplasmic portion of chB1r but not chB1 delivers inhibitory signals for BCR-induced NF-AT activation. (A) Schematic diagram of chB1–chB1r chimeric molecules. Open and shaded boxes represent portions of chB1 and chB1r, respectively. (B–G) NF-AT activity by BCR cross-linking and co-ligation of BCR with chB1/chB1r chimeric molecules in chB1r/chB1 double-deficient DT40 cells. chB1r/chB1 double-deficient DT40 cells were co-transfected with NF-AT-Luc plasmid and plasmids either encoding wild-type chB1 (B), chB1Y7F (C), chB1/chB1r (D), chB1/chB1r-Y7F (E), chB1/chB1r-Y38F (F) or chB1/chB1r-Y7/38F (G). After 48 h, cells were either left unstimulated (medium) or stimulated with anti-chicken IgM or anti-chB1 plus anti-IgM with (Fab')2 fragment of rabbit anti-mouse IgG for 6 h and subsequently assayed for luciferase activity. Cells were also stimulated by phorbol myristate acetate plus ionomycin (P + I) as a positive control. The NF-AT activity is expressed as the relative luciferase activity normalized by β-Gal activity. A representative of at least three independent experiments is shown. (H) Tyrosine phosphorylation of chB1–chB1r chimeric molecules upon cross-linking of BCR or BCR with chB1/chB1r chimeric molecules. chB1r/chB1 double-deficient DT40 cells transiently expressing chB1–chB1r chimeric molecules were stimulated for 5 min as in (B–G). Chimeric molecules were then immunoprecipitated with anti-T7 mAb and immunoblotted with anti-phosphotyrosine antibody PY20 (upper panels) or anti-T7 mAb (lower panels). Numbers below the upper panels indicate the induction levels of tyrosine phosphorylation over the unstimulated control, determined by densitometry after normalizing for loading.
ITIM2/Grb2 deliver a dominant growth inhibition and an additional growth-promoting signal, respectively (22).

CD72 is expressed throughout B cell development from pre-B to mature B cell stages except for plasma cells. The overall pattern of chB1r mRNA expression appears to parallel that of mouse CD72. Although chB1r expression is detectable in the thymus, its expression was undetectable in thymic T cells, including CD4+, CD8+ single-positive and CD4+CD8+ double-positive T cells, suggesting that minor cell populations other than T-lineage cells in the thymus express chB1r. Supporting to this, only B cell lines, but not T cell lines, express chB1r. The differential expression of chB1r and chB1, together with their functional differences, suggests that chB1r and chB1 play different roles in avian B cell development.

How do the two CD72 homologues function in chicken B cell development? Since chB1 does not interact with SHP-1 and fails to inhibit BCR-mediated signaling, chB1 might function as a decoy receptor that does not transduce signals by itself but captures ligands expressed in the bursa. If chB1r and chB1 share their ligands, competition of chB1 with chB1r in ligand binding might weaken chB1r-mediated inhibitory signals. Alternatively, chB1 might form a heterodimer or oligomer with chB1r to increase affinity for the ligands. Thus, chB1 may regulate either positively or negatively the chB1r-mediated modulation of the BCR threshold whose signal is necessary for proliferation and selection of diversified B cell repertoire in the bursa (23). To test these hypotheses, identification of the ligands for chB1r and chB1 is mandatory. In this regard, one of the semaphorin family proteins, CD100, has been identified as a ligand for mouse CD72 (24). Although the C-type lectin domain of chB1r, chB1 and CD72 is highly diverged, particularly in the C-terminal half, it will be necessary to determine whether chB1r and chB1 bind to the chicken CD100 homologue, in order to understand the physiological roles of chB1r and chB1 in avian B cell development.

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Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>BCR</td>
<td>B cell antigen receptor</td>
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<tr>
<td>GAPDH</td>
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